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High-level IL-10 production by monoclonal antibody-stimulated human T cells

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SUMMARY

We investigated interleukin-10 (IL-10) production in freshly isolated mononuclear cells and purified T cells in response to stimulation with monoclonal antibodies (mAb) recognizing CD3, CD2 and CD28, or with the bacterial products *Staphylococcus aureus* cells (SAC), staphylococcal enterotoxin (SEA) and lipopolysaccharide (LPS). IL-10 production was compared with that of IL-2, IL-4 and interferon-γ (IFN-γ). Similar to the other cytokines, in peripheral blood mononuclear cells (PBMC) from adult donors the highest IL-10 levels were produced in response to CD2 plus CD28 stimulation, within 72–96 hr of stimulation. Levels of IL-10 in response to CD2 plus CD28 stimulation (1·9 ± 1 ng/ml) exceeded those in response to SEA (0·25 ± 0·16 ng/ml), SAC (0·43 ± 0·42 ng/ml), or LPS (0·19 ± 0·14 ng/ml) stimulation. With adult purified T cells, high levels of IL-10 and IL-4 were measured following CD3 plus CD28 stimulation, and the amounts of both T-helper type-2 (Th2) cytokines decreased following the addition of phorbol myristate acetate (PMA), whereas the synthesis of the Th1 cytokines IL-2 and IFN-γ was enhanced. When PBMC were stimulated with a CD3 mAb and different other cytokines were added, strong enhancement of IL-10 production was seen upon the addition of IL-2, IL-4, IL-7, IL-12 and IFN-γ, whereas inhibition was found with transforming growth factor-β_1 (TGF-β_1). These data illustrate that in freshly isolated PBMC large amounts of IL-10 can be induced rapidly by appropriate mAb stimulation, and that even in freshly isolated cells IL-4 and IL-10 show signs of parallel regulation.

INTRODUCTION

The human immune system is composed of different cell types that communicate either by direct cell–cell contact or by the regulated synthesis and secretion of a number of cytokines, which influence the growth, differentiation and function of their target cells. T cells play a dominant role in this regulatory network of cytokines as they can synthesize many of them.

Interleukin-10 (IL-10) is a cytokine of 35 000–40 000 MW. Originally described as an inhibitor of cytokine synthesis of mouse T-helper type-1 (Th1) cells produced by Th2 cells,[1] it became clear subsequently that this factor could be synthesized by a variety of cell types, including activated B cells, monocytes and freshly isolated T cells.[2]

IL-10 inhibits T-cell activation and the synthesis of some T-cell derived cytokines, primarily by down-regulating certain costimulatory functions of monocytes, including the induction of the B7/BB1 antigen,[3,4] the major histocompatibility complex (MHC) class II molecule[5,6] and the intercellular adhesion molecule-1 (ICAM-1).[7] In addition, the production of T-cell stimulating cytokines [IL-1, IL-6, tumour necrosis factor-α (TNF-α) and IL-12] was shown to be inhibited by IL-10.[4,6,8,9] Finally, a direct inhibition of certain T-cell functions has also been observed.[10,12] On the other hand, IL-10 is a potent growth and differentiation factor for B lymphocytes.[13]

Although the effects of IL-10 on the function of T cells have been investigated by many studies, the regulation of its production is poorly understood. IL-10 is produced by activated B cells and by B cells following their transformation with Epstein–Barr virus (EBV).[14] Monocytes produce IL-10 when stimulated by lipopolysaccharide (LPS) or *Staphylococcus aureus* cells (SAC).[15,16] In monocytes the production of IL-10 is inhibited by interferon-γ (IFN-γ) and by IL-10 itself.[15,16]

Very little is known about the regulation of the production of IL-10 by human T cells. Yssel *et al.*[17] reported IL-10 production by both $CD4^+$ and $CD8^+$ freshly isolated T cells following CD3 and phorbol ester stimulation, and found the highest IL-10 levels to be produced by the $CD4^+$ $CD45RO^+$ memory subpopulation of helper T cells. We were interested in how T-cell stimulation through different surface molecules

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Abbreviations: SAC, *Staphylococcus aureus* cells; SEA, staphylococcal enterotoxin A.

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would modulate the IL-10 production by these cells. Here we report the cytokine pattern (IL-2, IL-4, IL-10, IFN-γ) of mononuclear cells and of T cells following stimulations by different monoclonal antibodies (mAb), bacterial antigens and phorbol ester, and its modulation by different cytokines.

MATERIALS AND METHODS

Cells and cell lines

Human peripheral blood mononuclear cells (PBMC) were prepared by isolation from buffy coats from healthy donors using Ficoll–Paque (Pharmacia, Uppsala, Sweden). The cells were enriched for $CD3^+$ T cells by passing them through a nylon wool column and by incubating them with antibodies directed against CD14 (Becton Dickinson, Mountain View, CA), CD16 (CLB-149; Jannsen, Geel, Belgium) and CD19 (Leu-12; Becton Dickinson) followed by extraction of marker positive cells with Dynabeads (Dynal, Oslo, Norway). The resulting cell population consisted of more than 98% $CD3^+$ T cells.

The HUT78 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI-1640 with 10% fetal calf serum (FCS). For stimulation experiments, the FCS was exchanged for human serum (5%).

Monoclonal antibodies and reagents

The following mAb were used in this study: CD3 (OKT3; IgG2a; Ortho, Raritan, NJ) immobilized at 10 μg/ml on rabbit anti-mouse immunobeads (Biorad, Hercules, CA; 100 000 beads/well), CD2 (OKT11; IgG1; ATCC; 500 ng/ml) plus VIT13 (IgM; Institute of Immunology, University of Vienna, 5 μg/ml) and CD28 (CLB-15E8; IgG1; kindly provided by Dr L. Aarden, CLN Amsterdam, the Netherlands; 500 ng/ml). Other stimulating reagents were phorbol myristate acetate (PMA; Sigma, St Louis, MO; 10^{-7} M), staphylococcal enterotoxin A (SEA; Sigma; 10 ng/ml), LPS (bacterial strain 0111:B4; Sigma; 1 μg/ml) and SAC (Sigma; 0·01%). Recombinant (r)IL-2 was kindly provided by Sandoz Research Institute (Vienna, Austria); rIL-1, rIL-4, rIL-6 and recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF) were provided by Sandoz AG (Basel, Switzerland). Recombinant TNF-α and rIFN-γ were kindly provided by Bender (Vienna, Austria). Recombinant IL-7 was obtained from Biosource International (Westlake Village, CA) and rIL-12 and transforming growth factor-β_1 (TGF-β_1) were purchased from British Biotechnology (Oxford, UK).

Cell stimulation and measurement of proliferation

Triplicates of 1×10^5 cells were cultured in U-bottomed 96-well plates (Nunc, Roskilde, Denmark) in RPMI-1640 containing 5% pooled human serum at 37° under 5% CO_2. Stimulating agents and antibodies were mixed prior to addition of cells. Seventy-eight hours after stimulation, 0·5 μCi of [^{3}H]thymidine (Du Pont, Boston, MA) was added to each well and the incorporated radioactivity was measured in a β-counter.

Measurement of cytokine production

For measurement of the cytokine production of T cells, the supernatants of triplicate cultures identical to those described above were harvested after 72 hr (if not otherwise stated) and the amount of each cytokine was assessed by classical sandwich enzyme-linked immunosorbent assay (ELISA) with different mAb for coating and detection. The ELISA for IL-4 was kindly provided by Dr F. Di Padova (Sandoz, AG) and uses mAb 1-41-1 for coating and 1-38-10 for detection. The ELISA for IL-2 was established using mAb IL2-13C2 (Institute of Immunology, University of Vienna) for coating and mAb IL2-1F10 (Institute of Immunology, University of Vienna) for detection. The IFN-γ ELISA was established using mAb 1598-00 (Genzyme, Cambridge, MA) for coating and mAb GZ-4 (kindly provided by Dr M. Schaude, Bender) for detection. The mAb used for the IL-10-specific ELISA were JES3–9D7 for coating and the biotinylated JES3–12G8 for detection (Pharmingen, San Diego, CA). Recombinant IL-10 for standardization of the ELISA assay was kindly provided by DNAX (Palo Alto, CA). In each case coating was performed over night in phosphate-buffered saline (PBS) at 4°; for blocking non-specific binding a 2% solution of bovine serum albumin (BSA; no. 0812; Behring, Vienna, Austria) in PBS was used subsequently for 4 hr. Standard and test samples were incubated overnight at room temperature. The detection antibody (incubation time 4 hr at room temperature) was biotinylated and its binding was detected by streptavidin-conjugated alkaline phosphatase (Chemicon, Temecula, CA; incubation time 1 hr).

RESULTS

IL-10 production by human mononuclear cells

To investigate the IL-10 production by human PBMC, we measured IL-10 in the supernatant of such cells along with other typical T-cell derived cytokines (IL-2, IL-4, IFN-γ) following cell stimulation by means of mAb recognizing distinct T-cell signal transducing molecules (CD3, CD2, CD28). In parallel cultures, we stimulated the cells with bacterial-derived antigens (SEA, SAC, LPS) or with the phorbol ester PMA. The results of these experiments are shown in Table 1.

As a general feature of these experiments, we noted a huge variation between individual cell donors for their capacity to produce various cytokines, although the cell proliferation values varied much less between different donors. This was reflected by the high numbers for the standard deviation (SD) for mean cytokine levels. Nevertheless, mean cytokine numbers still reflected well the relative cytokine production when comparing different cell stimuli, because most individual donors behaved in this respect with a similar pattern: Stimulating T cells via the T-cell receptor (TCR)/CD3 complex induced only low levels of IL-10. Cross-linking of the CD3 complex by OKT3 presented on beads stimulated a mean release of 89 ± 74 pg/ml of IL-10. A pair of stimulating CD2 antibodies (mAb OKT11 and VIT13) stimulated five times higher amounts of IL-10 (495 ± 491 pg/ml) compared to a CD3 stimulation. Costimulation through the CD28 antigen was found to enhance the IL-10 release by three- to seven-fold, depending on the primary stimulus used. By far the highest levels of IL-10 were induced by stimulating T cells through both the CD2 and the CD28 activation pathways (1864 ± 1035 pg/ml). All other cytokines measured in this study showed a similar pattern of production by PBMC, depending on mAb stimulation (Table 1).

In contrast, the proliferative response of the cells differed much less in response to the different stimuli. Stimulation of

Table 1. Mean of cytokine production of PBMC after stimulation with different activating reagents ($n = 5$)

	IL-2 (pg/ml ± SD)	IL-4 (pg/ml ± SD)	IL-10 (pg/ml ± SD)	IFN-γ (ng/ml ± SD)	Proliferation (c.p.m. ± SD) ($\times 10^{-3}$)
Medium	34·8 ± 51·9	ND	38·0 ± 39·1	ND	2·1 ± 5·5
CD3B	ND	1·2 ± 2·7	89·0 ± 74·5	4·1 ± 3·5	70·2 ± 20·6
CD2	ND	18·2 ± 21·9	494·6 ± 491·0	42·9 ± 45·7	99·7 ± 21·8
PMA	ND	4·4 ± 9·8	43·8 ± 74·5	4·7 ± 8·4	94·4 ± 29·4
CD28	ND	ND	9·8 ± 13·8	6·6 ± 14·8	1·7 ± 1·9
CD3B + CD28	253·6 ± 317·0	38·4 ± 72·6	641·8 ± 629·3	72·6 ± 50·3	170·5 ± 28·1
CD2 + CD28	1124·4 ± 1114·5	162·4 ± 135·8	1864·0 ± 1035·6	129·4 ± 55·7	169·7 ± 43·4
PMA + CD28	101·0 ± 69·7	27·2 ± 40·6	141·6 ± 80·5	40·7 ± 43·2	163·4 ± 10·9
SAC	7·4 ± 16·5	ND	432·2 ± 422·7	1·8 ± 1·0	10·1 ± 4·5
SEA	73·8 ± 103·6	17·2 ± 27·7	253·0 ± 157·8	25·9 ± 19·6	119·3 ± 16·9
LPS	9·6 ± 21·5	4·0 ± 8·9	186·0 ± 142·5	0·5 ± 0·4	3·6 ± 0·9

ND, not detectable in any experiment.

Levels of sensitivity for IL-2 were 25 pg/ml; IL-4, 4 pg/ml; IL-10, 5 pg/ml; IFN-γ, 0·5 ng/ml; for calculation of mean values measurements below the level of sensitivity in single experiments were arbitrarily set to 0.

PBMC with either the CD2 mAb pair, CD3 beads or PMA resulted in an average proliferation of between 70 000 and 100 000 c.p.m., which could be enhanced by simultaneous CD28 stimulation (Table 1).

Addition of SAC to mononuclear cells induced IL-10 levels of around 0·5 ng/ml (432 ± 422 pg/ml). However, none of the other cytokines (IL-2, IL-4, IFN-γ) nor substantial cell growth was induced (Table 1). SAC has been described as stimulating both B cells and monocytes, which are well known for their IL-10-releasing capacity.[15] Another B-cell and monocyte-activating compound is LPS.[18] In our experiments LPS induced mean levels of 186 pg/ml of IL-10, which are low levels compared to stimulation with T-cell specific mAb (Table 1). As Malefyt *et al.*[16] reported the production of high levels of IL-10 by PBMC stimulated with LPS, we tested LPS obtained from different *Escherichia coli* strains at various concentrations. However, IL-10 production in response to this agent remained relatively low in this culture system (data not shown).

The superantigen SEA was the only bacterial antigen that could induce the production of IL-10, together with the T-cell prototypic cytokines (IL-2, IL-4 and IFN-γ). Again the release of IL-10 was low compared to mAb stimulation (Table 1).

Following stimulation with CD3 beads, alone or in combination with a CD28 mAb, peak IL-10 levels were reached between 72 and 96 hr. CD2 plus CD28 stimulation had similar time kinetics, with much higher absolute levels of IL-10, whereas CD2 stimulation alone resulted in a delayed time kinetics of IL-10 production (Fig. 1).

IL-10 production is strongly influenced by the presence or absence of accessory cells in a stimulus dependent way

The experiments conducted so far were performed with unfractionated mononuclear cells, which are composed of different cellular fractions at frequencies differing from cell donor to donor. In order to investigate whether differences in the number of non-T cells would influence cytokine production by stimulated T cells in general and IL-10 production in particular, highly purified T cells were stimulated with CD3, CD3 plus CD28 or CD2 plus CD28 in the absence or presence of graded numbers of sheep erythrocyte-rosette$^-$ (E^-) non-T cells.

IL-10 and IL-4 were very efficiently induced when purified T cells were stimulated with CD3 beads together with a CD28 mAb (≈ 1 ng/ml IL-10, ≈ 1 ng/ml IL-4; Fig. 2, lower panels). In contrast to cultures with PBMC, with purified T cells these stimuli were much more potent than a CD2 plus CD28 stimulation, which served as the most potent inducer of IL-10 in the mixed population of PBMC (Fig. 2 and Table 1), but induced less than 100 pg/ml of IL-10 in purified T cells (Fig. 2). When increasing numbers of accessory cells were added to the culture stimulated with CD3 beads, a dramatic decrease in the release of IL-4 and IL-10 was observed. In contrast, optimal

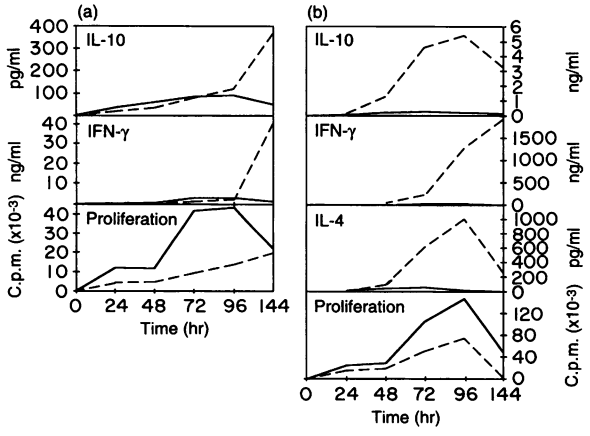

Figure 1. Kinetics of cytokine production by human PBMC. Mononuclear cells were either stimulated with CD3 beads (solid line) or a pair of stimulating CD2 mAb (dashed line) in the absence (a) or in the presence (b) of a CD28 mAb. In the absence of the CD28 mAb no IL-4 could be measured, therefore the corresponding panel is omitted from (a). Similar data were obtained in a second experiment.

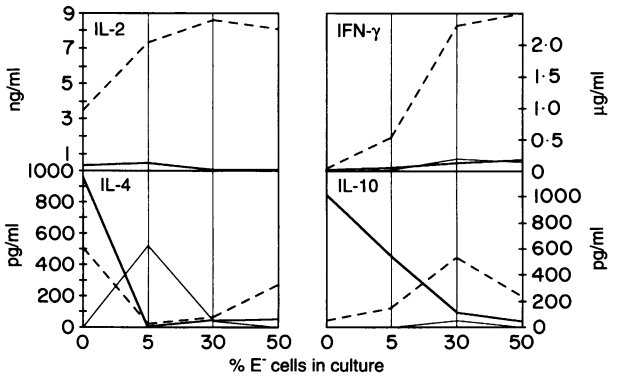

Figure 2. Antigen-presenting cells modulate the release of IL-10 from human T cells. Purified T cells were stimulated with different numbers of E^- cells for 72 hr. The percentage of E^- cells from the number of T cells is indicated on the abscissa. The cells were stimulated with a soluble CD3 mAb plus a CD28 mAb (thin line) or CD3 beads plus a CD28 mAb (solid line), or a CD2 mAb pair plus a CD28 mAb (dashed line). The results shown are representative data from one of three independent experiments.

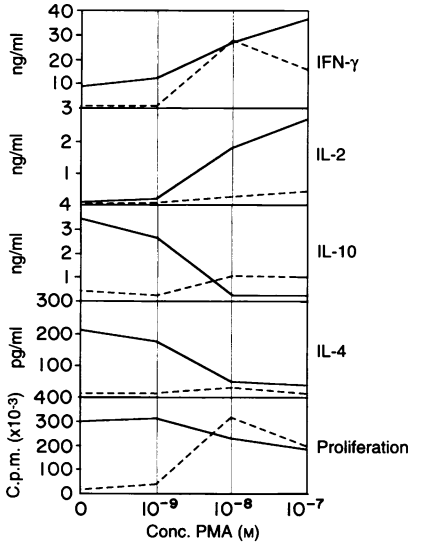

Figure 3. Influence of phorbol esters on the production of IL-10. Purified T cells were stimulated either with CD3 beads alone (dashed line) or with CD3 beads plus a CD28 mAb (solid line) for 72 hr. PMA was added in increasing concentrations as indicated. The experiment shown is representative of three independent experiments. PMA alone in the absence of any other stimulation induced only marginal levels of cytokines or cell proliferation in purified T cells (for simplicity these data are not shown in this figure).

amounts of IL-10 were released in the presence of 30% E^- cells after CD2 plus CD28 stimulation.

The production of IFN-γ was strongly enhanced by the presence of accessory cells independent of the stimulus used, whereas enhancement of IL-2 production by accessory cells was seen only for CD2 plus CD28-stimulated cells (Fig. 2).

Increasing concentrations of phorbol esters inhibit the generation of IL-10

Pouw-Kraan *et al.*[19] recently reported differences in the regulation of IL-4 versus IL-2 production by the phorbol ester PMA. To test the influence of PMA on the production of IL-10, purified T cells were stimulated either with CD3 beads or with a combination of CD3 beads and a CD28 mAb, and PMA was added between 10^{-9} to 10^{-7} molar concentration (Fig. 3).

When cells were stimulated with CD3 beads alone, the addition of PMA at a concentration of 10^{-8} M was optimal for the induction of all cytokines as well as the proliferative response of the cells (Fig. 3, dashed lines). Therefore, with CD3 beads as a primary stimulus, we did not see a differential regulation of the different cytokines by the phorbol ester.

In contrast, a stimulation of T cells by a combined triggering of CD3 plus CD28 led to a totally opposite pattern of cytokines when PMA was added (Fig. 3). The release of the Th1 cytokines IL-2 and IFN-γ was strongly augmented by the addition of increasing amounts of the phorbol ester PMA. At least a fivefold induction of the cytokine response was noted using 10^{-7} M PMA. On the other hand, the two Th2 cytokines IL-4 and IL-10 were influenced in the opposite way by the presence of PMA. Increasing concentrations of PMA efficiently reduced the release of these cytokines. Notably, the proliferation of the cells was also diminished in parallel, although very high levels of the major T-cell growth factor, IL-2, were present in these samples.

Cord blood T cells synthesize only low levels of IL-10

Naive T cells derived from cord blood were shown to be unable to synthesize cytokines other than IL-2. Only following an activation and differentiation step did such cells produce IL-3, IL-4, IL-5, IL-6, IFN-γ and GM-CSF.[20] We analysed whether IL-10 belongs to the group of cytokines solely produced by differentiated T cells. This seemed to be the case, as not even the strongest stimulus for IL-10 production by PBMC, a combination of CD2 plus CD28 mAb, nor any other mAb combination induced the production of amounts of IL-10 comparable to those released by peripheral blood cells from adult donors (maximal amount 180 pg/ml after CD3 beads plus CD28 stimulation; Table 2). In contrast, the proliferative rate of the cord blood cells reached levels comparable to those obtained with adult donors, when cells were stimulated with CD3 beads with or without CD28 mAb (170 000–330 000 and 20 000–48 000 c.p.m., respectively; Table 2).

The T-cell stimulating cytokines IL-2, IL-4, IL-7, IL-12 and IFN-γ stimulate the production of IL-10

To investigate the effect of exogeneously added cytokines on the generation of IL-10, PBMC were either stimulated with T-cell specific mAb or the bacterial antigen SAC, in the presence or absence of various cytokines that are known to influence T-cell responses (Table 3). The cytokines IL-2, IL-4, IL-7, IL-12 and IFN-γ significantly enhanced the production of IL-10 by PBMC stimulated with CD3 beads. The most prominent helper factor for IL-10 production was IL-2, which led to a more than eightfold induction of IL-10. The presence of IL-4, IL-7 or IL-12 at least tripled the amount of IL-10 produced by the cells. IFN-γ still doubled the amount of IL-10 stimulated by CD3 beads.

A less pronounced enhancement of IL-10 production by added cytokines was observed when the cells were stimulated

Table 2. Cytokine production and proliferation of mononuclear cord blood cells

		IL-10 (pg/ml)	IFN-γ (ng/ml)	Proliferation (c.p.m.)
Donor A	Medium	19	ND	2·8
	CD3B + CD28	46	1·4	328·1
	CD2 + CD28	18	1·3	83·8
Donor B	Medium	29	ND	4·4
	CD3B87	0·3	20·3	
	CD2 20	ND	2·7	
	CD3B + CD28	180	2·5	223·6
	CD2 + CD28	132	50	186
Donor C	Medium	17	ND	1·9
	CD3B	56	0·7	48·2
	CD2 18	ND	1·5	
	CD3B + CD28	117	6·1	169·3
	CD2 + CD28	44	5·1	72·7

Mononuclear cord blood cells were stimulated as indicated and supernatants harvested after 72 hr were analysed for IL-10 and IFN-γ, whereas cell proliferation was assayed after 96 hr following an 18-hr labeling period.

ND, not detectable.

CD3B, bead-coated CD3 mAb.

with stronger stimuli, such as CD3 beads and a CD28 mAb or the combination of CD2 mAb plus CD28 mAb. We speculate that in these cultures high amounts of endogeneously produced cytokines (e.g. of IL-2) could be the cause for the relatively small enhancement of IL-10 production by exogenously added cytokines (Table 3).

Following stimulation of PBMC with SAC, no enhancing effects were observed of any cytokines tested in this experiment on their IL-10 production (Table 3). As SAC mainly activates monocytes and B cells,[15] these results support the hypothesis that the effects of the cytokines were specific for the IL-10 production by T cells.

TGF-β_1 reduces IL-10 production induced by various T-cell stimuli

In the above experiments a consistent inhibition of IL-10 production was noticed in the presence of TGF-β_1. Dependent on the stimulus used, the production of IL-10 was reduced to between 34% and 58% of the response in the absence of TGF-β_1 (Table 3). In an earlier study we had found that TGF-β_1 reduced the production of IL-2-dependent cytokines such as IL-4 and IFN-γ, possibly by inhibition of the signal transduction of the IL-2 receptor.[21] In view of the super-induction of IL-10 by IL-2 and its inhibition by TGF-β_1 (Table 3), we investigated the effect of the combined addition of IL-2 plus TGF-β_1 on T-cell cytokine production, including IL-10 synthesis (Table 4). In this experiment we again found that a clear antagonism existed between IL-2 and TGF-β_1 (Table 4).

The HUT78 cell line as a model system for studying IL-10 production

We have previously used the human T-cell line HUT78 for studying the regulation of different cytokines.[22] When this cell line was tested for IL-10 production, a high amount of spontaneously produced IL-10 was found (Fig. 4). This is the first cytokine we have found consistently in the culture medium of unstimulated HUT78 cells.

Stimulation of the cell line either with a CD3 mAb plus

Table 3. Influence of cytokines on the production of IL-10 by PBMC

	CD3 Beads (n = 3) (% production)	CD3B/CD28 (n = 3) (% production)	CD2/CD28 (n = 6) (% production)	SAC (n = 3) (% production)
Medium	100	100	100	100
IL-1	118·7 ± 20·6	114·5 ± 1·6	110·5 ± 46·4	54·6 ± 5·3*
IL-2	851·8 ± 311·7**	143·9 ± 11·0*	143·3 ± 77·6	48·1 ± 20·1*
IL-4	333·4 ± 268·9*	180·1 ± 10·7*	171·3 ± 42·0	47·6 ± 7·3*
IL-6	81·4 ± 1·3	124·0 ± 7·6	127·8 ± 27·8	48·2 ± 7·0*
IL-7	427·1 ± 36·6*	177·3 ± 46·7*	164·1 ± 65·3	109·7 ± 41·4
IL-12	391·5 ± 261·6*	105·4 ± 17·1	146·2 ± 61·2	58·8 ± 26·9*
IFN-γ	196·8 ± 162·6*	124·9 ± 8·8	152·7 ± 51·3	77·6 ± 15·0
TNF-α	81·0 ± 20·2	106·8 ± 0·9	108·5 ± 38·4	46·9 ± 7·9*
TGF-β	58·0 ± 51·9	34·4 ± 12·6*	50·2 ± 27·3*	84·3 ± 2·2

Mononuclear cells were stimulated for 72 hr and supernatants were analysed for IL-10 by ELISA. Within each experiment the release of IL-10 after mAb or SAC stimulation was set to 100% and the changes in response to the addition of the various cytokines were normalized to this value. Shown are the mean ± SD of the percentages of change calculated from three to six experiments.

The cytokines were used in the following concentrations: IL-1, IL-4, IL-6, IL-7, TNF-α and GM-CSF, 100 U/ml; IL-2 and IL-12, 10 ng/ml; IFN-γ, 50 ng/ml; TGF-β_1, 10 ng/ml.

Significance according to paired t-test: *$P < 0.05$; **$P < 0.01$. Mean ± SD of IL-10 production was for CD3 beads, 110 ± 83 pg/ml; for CD3/CD28, 183 ± 122 pg/ml; for CD2/CD28, 1377 ± 1230 pg/ml; for SAC, 328 ± 286 pg/ml.

Table 4. Cytokine production of PBMC in the presence of TGF-β and/or IL-2

		Medium	TGF-β_1	TGF-β_1 + IL-2	IL-2
IL-2 (pg/ml)	Medium	ND	ND	—	—
	CD3B	ND	ND	—	—
	CD3B + CD28	ND	ND	—	—
	CD2 + CD28	781	584	—	—
IL-4 (pg/ml)	Medium	ND	ND	ND	ND
	CD3B	8·4	ND	11·8	39·4
	CD3B + CD28	26·8	ND	24·8	177·6
	CD2 + CD28	57·4	13·1	19·9	44·8
IL-10 (pg/ml)	Medium	61	43	ND	71
	CD3B	312	45	398	2816
	CD3B + CD28	70	84	32	1742
	CD2 + CD28	275	175	227	738
IFN-γ (ng/ml)	Medium	1·9	0·16	0·37	7·19
	CD3B	13	3·08	44·39	91·68
	CD3B + CD28	10·97	0·77	47·6	52·11
	CD2 + CD28	92·09	53	50·69	148·68

ND, not detectable.
— Not measured.

PMA or a CD3 mAb coated to the culture plate induced a 3·9- and 3·2-fold enhancement of IL-10 production, respectively (Fig. 4). The addition of a high dose of IL-2 further increased the release of IL-10 to more than six times the level by a cross-linked CD3 mAb alone. On the other hand, inhibitory effects of TGF-β_1 on the stimulated production of IL-10 were consistently found, both in the presence and in the absence of exogenous IL-2. In a dose-dependent manner TGF-β_1 inhibited the generation of IL-10 by up to 50% for the stimulation of CD3 plus PMA, and by up to 76% in the case of cross-linked CD3 mAb.

DISCUSSION

In this study we show that human PBMC can be induced to release high levels of IL-10 by a number of different stimuli. The highest levels of IL-10 were produced when the cells were stimulated with a pair of CD2 mAb together with a CD28 mAb (Table 1). CD2 mAb, alone or in combination with a CD3 mAb coupled to beads plus a CD28 mAb, also induced substantial production of IL-10. Interestingly, CD2 stimulation induced more than five times more of IL-10 than a stimulation via the classical CD3 pathway. It is important to note, however, that these results were obtained with unfractionated mononuclear cells. When purified T cells were studied, stimulation with CD3 beads plus a CD28 mAb was the most efficient way to induce IL-10 production (Fig. 2). Together these data illustrate the complexity of cytokine production in commonly used *in vitro* test systems and underline the necessity of carefully standardizing such assays.

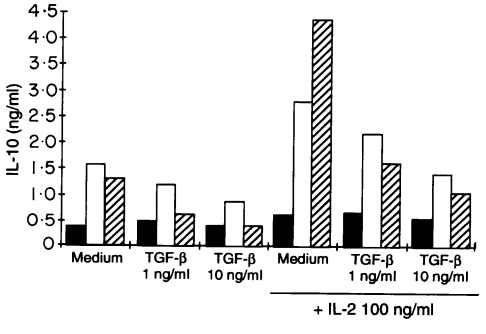

Figure 4. Influence of TGF-β_1 on IL-10 production of the human T-cell line HUT78. The HUT78 cell line was either left unstimulated (black bars) or stimulated with OKT3 mAb plus PMA (white bars) or OKT3 mAb coated onto the wells (striped bars). Cytokines were added as indicated. The supernatants of the cells were harvested 48 hr after onset of the culture.

The pattern of IL-10 production by human T cells paralleled the production of other important T-cell derived cytokines, namely that of IL-4 and of IFN-γ. They can all be induced by triggering either the CD3 or the CD2 antigen and are superinduced by a CD28 mAb. Regarding IL-2 production, we were not able to induce measurable amounts of IL-2 by stimulating the CD3 or the CD2 antigen alone. Co-engagement of the CD28 antigen was required for an efficient induction of this cytokine. It is known that CD28 co-stimulation enhances the production of IL-2 primarily by up-regulating the half-life of the IL-2 mRNA. It is possible that the enhancing effects on other cytokines are at least in part mediated via IL-2,[23] in particular as we have shown that IL-10 production is strongly enhanced by IL-2 under certain circumstances (Table 3).

In the murine system IL-10 is produced solely by Th2 cells. Human IL-10 has been reported not to be restricted to Th2 cells.[12,17] We found, however, some similar features between IL-4 and IL-10, which contrast with those typical for IL-2 and IFN-γ in our system. First, IL-4 and IL-10 were both inhibited by increasing concentrations of the phorbol ester PMA

(Fig. 3). Pouw-Kraan *et al.*[19] observed this phenomenon initially for IL-4. In contrast, IL-2 and IFN-γ both showed a marked enhancement of their production in the presence of PMA (Fig. 3).

Second, IL-4 and IL-10, but not IFN-γ, were strongly inhibited by the presence of non-T cells in mixed cell cultures, when the cells were stimulated by CD3 beads. Because this phenomenon was not seen with all cytokines, a toxic effect mediated by accessory cells is unlikely. We speculate that some inhibitory factors, either soluble or cell bound, were induced in these cultures by CD3 beads in the presence of E^- cells, possibly by triggering of monocytic Fc receptors by OKT3.[24] This putative inhibitory factor down-regulated IL-4 and IL-10, but not IFN-γ.

The cytokines IL-2, IL-4, IL-7, IL-12 and IFN-γ significantly enhanced the IL-10 production of mononuclear cells prestimulated with T-cell specific mAb (Table 3), whereas inhibition of IL-10 synthesis was observed for TGF-β_1, both with PBMC (Table 3) as well as with the malignant-T cell line HUT78 (Fig. 4). Other cytokines that were inhibited by TGF-β_1 included some whose synthesis is dependent on IL-2, such as IL-4 and IFN-γ,[21] and others for which at least enhancement of synthesis by IL-2 was demonstrated, such as IL-10 (Table 4). TGF-β_1 inhibits signal transduction through the IL-2 receptor,[21,25,26] and by this means probably interferes with the synthesis of IL-2-dependent cytokines. This model is also supported by the observation that TGF-β_1 hardly influenced IL-10 production by unstimulated HUT78 cells, which do not spontaneously secrete IL-2.[22] Finally, it is noteworthy that TGF-β_1 inhibited IL-10 production by stimulated T cells and by HUT78 even in the presence of high doses of IL-2 (Table 4 and Fig. 4).

We have demonstrated that IL-10 is present in many cultures of PBMC activated differentially *in vitro*. Our data show that the amount of IL-10 synthesized is strongly dependent on a number of variables, such as the nature of the T-cell surface antigen triggered (Table 1), the number of E^- cells in the proximity of the T cells (Fig. 2) and the amounts of a number of cytokines present (Tables 3 and 4). All these factors are also variables under *in vivo* conditions. Our data, therefore, identify IL-10, which has strong immunoregulatory functions, as a critically regulated key cytokine that is present early following immune cell activation.

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